

Separation of γ -Casein²

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Mellander³ observed that casein was composed of three electrophoretic components, which he named α -, β - and γ -casein in the order of decreasing mobility. He reported that the γ -casein component has a low phosphorus content, which suggests that γ -casein is similar to the alcohol-soluble, low-phosphorus casein isolated by Osborne and Wakeman.⁴ A method has been devised for separating γ -casein that consists in fractionation with 50% ethyl alcohol and with

water at pH 5.8. The γ -casein prepared in this manner has an isoelectric point of pH 5.8-6.0 and is electrophoretically homogeneous in solutions alkaline to the isoelectric point but inhomogeneous in acid solutions. The similarity between the composition and properties of γ -casein and those of the alcohol-soluble casein described by Osborne and Wakeman⁴ indicates that the two caseins are essentially the same.

Experimental

Preliminary experiments on the solubility of unfractionated casein in water-alcohol solution showed that under a given set of conditions solubility was greatest at an alcohol concentration of about 50%. The temperature, pH and salt content also affected its solubility in alcohol. Thus, a 20% solution of casein could be made at pH 4.7 in 50% alcohol that contained a high concentration of in-

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented before the Division of Biological Chemistry at the 117th Meeting of the American Chemical Society, Philadelphia, April, 1950.

(3) Mellander, *Biochem. Z.*, **300**, 240 (1939).

(4) Osborne and Wakeman, *J. Biol. Chem.*, **33**, 243 (1918).

organic salts such as ammonium nitrate. By controlling the pH, temperature and salt concentration, the components of casein were separated readily by differential solubility in 50% alcohol. The method described here for the separation of γ -casein is based in part on its solubility at room temperature in a 50% alcohol solution having a pH of 5.7 and containing low concentrations of salt. Under these conditions, α -casein and β -casein are largely insoluble. The progress of fractionation was followed by the Tiselius electrophoresis apparatus. Areas and mobilities were calculated by the method described by Warner.⁵

Measurements of pH in water-alcohol solutions were made with the glass electrode in the usual manner. The pH value of an aqueous casein solution was increased by about one unit by diluting it with alcohol to a 50% water-alcohol solution. No theoretical significance is attached to the pH values in 50% alcohol. The values have empirical significance, however, and the procedures based on these values are reproducible. The point of minimum solubility of unfractionated casein in 50% alcohol is at approximately pH 5.7, as compared with 4.7 in water.

In dissolving casein, considerable care was taken to prevent local excess of alkali. This was accomplished by diluting the alkali and adding it slowly with stirring to the casein suspended in water. The alkalinity of casein solutions in water did not exceed pH 8.0.

Preparation of γ -Casein.—The casein fraction of unpasteurized bovine skim milk was precipitated by adding the required amount of approximately normal hydrochloric acid to give a pH of 4.5. The precipitated casein was removed by filtration through a closely woven cotton bag. The casein from 15 gallons of milk, amounting to about 1600 g. (dry weight), was washed four times by suspending it in 20 l. of distilled water. The washed casein was further purified by two isoelectric reprecipitations from solutions made by dissolving it with dilute sodium hydroxide and adjusting to 28 l., then adding dilute hydrochloric acid until pH 4.7 was obtained.

To separate the fraction soluble in 50% alcohol, the casein was then dissolved in 925 cc. of *N* sodium hydroxide, giving a total volume of 15.4 l. with a pH of about 7. An equal volume of absolute ethyl alcohol was added slowly with stirring. The major portion of the α - and β -casein was precipitated by slowly adding about 600 cc. of *N* hydrochloric acid in 50% alcohol until the solution was reduced to pH 5.7. The precipitate was removed by filtration through fluted paper and washed with a total of 700 cc. of 50% alcohol. The filtrate was cooled to 2°, and the insoluble material, which contained the slow-moving electrophoretic component in high concentration, was removed by gravity filtration. After it was dried with acetone at 2° and ether at room temperature, the fraction weighed 68 and contained approximately 44% γ -casein (Fig. 1 (b)).

The fraction insoluble in 50% alcohol at room temperature was extracted again by dissolving and reprecipitating it in 31 l. of 50% alcohol at pH 5.7. The fraction soluble at room temperature but insoluble at 2° weighed 34 g. and contained approximately 29% γ -casein. The results obtained on re-extraction of the casein fraction insoluble in 50% alcohol at room temperature indicate that γ -casein was largely removed by the first extraction. The fraction rich in γ -casein (102 g.), obtained by the first and second extractions with 50% alcohol, was dissolved in a minimum amount of alkali and diluted to 2 l. An equal volume of absolute alcohol was then added, and the pH was adjusted to 5.7 by adding dilute hydrochloric acid in 50% alcohol. The precipitate was removed by centrifugation, and the supernatant was cooled to 2°. The fraction insoluble at 2° was removed by centrifugation at 2° and dried with acetone and ether at 2°. This fraction (20 g.) contained 64% γ -casein, as shown by the electrophoretic pattern in Fig. 1 (c). A further yield of 8 g. with the same composition was obtained by reworking the fraction insoluble in 50% alcohol at room temperature.

The remaining small amount of α - and β -casein was completely removed from the γ -casein by isoelectric pre-

cipitations at 2°. Twenty grams of the purified γ -casein was dissolved in the minimum amount of dilute sodium hydroxide and diluted to 1.5 l. The solution was cooled to 2°, and the pH adjusted to 4.7. The precipitate that contained α - and β -casein was removed by filtration at 2°; when the filtrate was adjusted to pH 5.8, and then warmed to 30°, γ -casein was precipitated. By re-extracting the precipitate insoluble at pH 4.7 and 2°, and reworking the combined product, a total of 8 g. of γ -casein was obtained. The electrophoretic pattern of γ -casein, free of α - and β -casein, is shown in Fig. 1 (d).

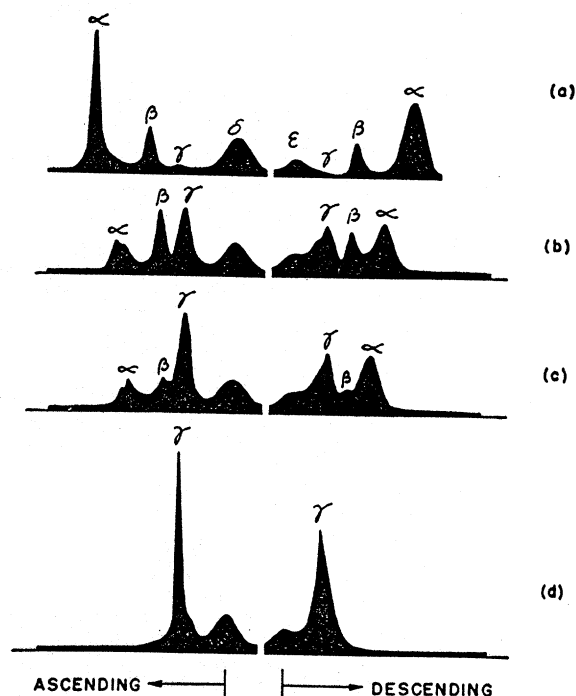


Fig. 1.—Electrophoretic patterns obtained in a veronal buffer (at pH 8.40; an ionic strength of 0.1; containing 0.05 *M* sodium chloride) with a protein concentration of 1%, after electrophoresis for 3 hours: (a) unfractionated casein at field strength of 4.73 volts/cm.; (b) first γ -fraction at field strength of 4.52 volts/cm.; (c) second γ -fraction at field strength of 4.61 volts/cm.; (d) γ -casein at field strength of 4.72 volt/cm.

Properties of γ -Casein.—Unlike α - or β -casein, γ -casein is a viscous, oily material when it is precipitated from water, and it is not readily dehydrated by the usual procedures. Dehydration by freeze-drying yields a hard glossy product. A fine-powdered product is obtained by dissolving the γ -casein in 50% alcohol at room temperature and then precipitating it by cooling to 2°. After centrifuging, the product is dehydrated at 2° with acetone and then with an equal mixture of acetone and ether. The 50% alcohol solution can be dehydrated also by careful dialysis against absolute alcohol.

Table I gives analytical data on γ -casein and some of its properties. Values for α - and β -casein are given for comparison; some are taken from Warner,⁵ and the remainder are new data.

In physical properties, γ -casein resembles β -casein rather than α -casein. There are distinct differences, however, in their phosphorus and sulfur contents and isoelectric points. The lower phosphorus content of γ -casein is reflected in its lower mobility. The extinction coefficient values and the nature of the absorption curve in the ultraviolet region for γ -casein as compared with

(5) Warner, *THIS JOURNAL*, 66, 1725 (1944).

TABLE I
COMPOSITION AND PROPERTIES OF COMPONENTS OF CASEIN

	α -Casein	β -Casein	γ -Casein
N, %	15.56	15.39	15.40
P, %	0.98	0.55	0.11
S, %	0.72	0.86	1.03
True ash, %	0.28	0.31	0.15
Mobility, μ ^c	-6.75	-3.05	-2.01
Isoelectric point, pH ^d	4.7	4.9	5.8-6.0
Specific rotation $[\alpha]_{25}^D$	-90.5	-125.2	-131.9
Extinction coefficient, K, at 278 m μ ^f	1.025	0.475	0.50
Clotting with rennet	+	+	Coagulates with Ca ⁺⁺ ions

^a Moisture-free basis. ^b Ash determinations were made in the presence of a known amount of calcium acetate. The value given was obtained by subtracting the weight of added calcium oxide and the theoretical weight of phosphorus pentoxide formed from the casein from the total weight of ash. ^c Veronal buffer pH 8.4, $\mu = 0.1$, containing 0.05 M NaCl, 1% solution. ^d As determined from the pH of minimum solubility at 25°. Warner⁶ has reported lower isoelectric points for α -casein and β -casein as determined by electrophoretic mobilities. ^e Determined on 1% solution in veronal buffer, pH 8.4, $\mu = 0.1$. ^f Specific extinction coefficient defined as the density for a 1-cm. layer of solution containing 1 g. per liter. Determinations were made at pH 7.2-9.4.

those of β -casein indicate somewhat higher tyrosine, tryptophan and phenylalanine contents for γ -casein.

Solubility.—A striking difference between the solubility of γ -casein and that of α - and β -casein in water and in 50% alcohol is shown in Table II. Solubilities were determined by the method of Linderström-Lang and Kodama.⁶ The determinations were made in an empirical manner by dissolving 0.1 g. of casein in dilute sodium hydroxide. Sodium chloride was added to give a final concentration of

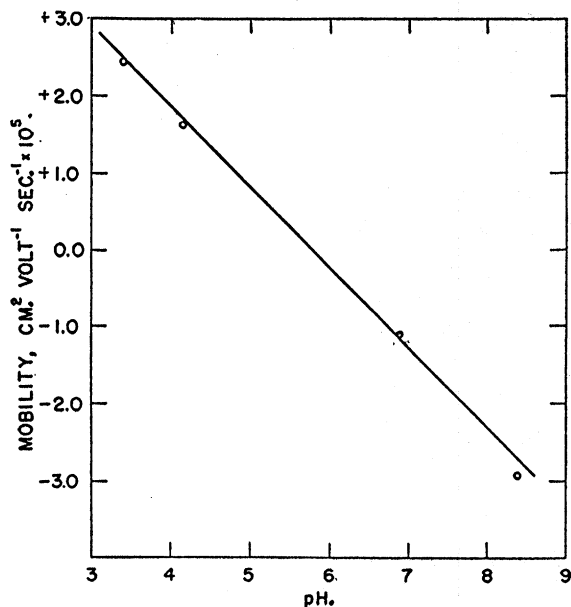


Fig. 2.—Effect of pH on mobility of γ -casein at 0.7° at an ionic strength of 0.05 and at a protein concentration of 0.4-0.5%. Veronal buffer was used at pH 8.4; phosphate buffer at pH 6.9; and lactate buffer at pH 4.15 and 3.4.

(6) Linderström-Lang and Kodama, *Compt. rend. trav. lab. Carlsberg*, 16, No. 1 (1925).

0.007 M, followed by addition of the desired amount of alcohol or water to about 90 cc. The required amount of acid was then added with shaking to give the desired pH. The volume was finally adjusted to 100 cc. The solutions were rotated at the desired temperature, and samples were removed periodically. The solubility was determined by the nitrogen content of the supernatant after centrifugation.

TABLE II
SOLUBILITY OF THE COMPONENTS OF CASEIN IN WATER AND IN 50% ETHYL ALCOHOL

Casein	pH	In water		pH ^a	In 50% alcohol by volume	
		Mg. N/100 cc. 25°	2.5°		Mg. N/100 cc. 25°	2.5°
α -	4.7	0.05 ^b	5.6	0.135	0.120
β -	4.7	0.41	8.46 ^c	5.6	1.90	.75
γ -	5.95	1.0	15.3 ^d	6.2	14.5	.88
γ -	5.3	2.83			

^a The pH values are the points of approximate minimum solubility in 50% alcohol. ^b Value with no sodium chloride added. ^c Value for 17 hr., which decreased to an equilibrium value of 1.50 after 161 hr. ^d Complete solution at 0.1% concentration.

γ -Casein is more soluble than α - and β -casein in water and in 50% alcohol at room temperature and at 2.5°. At its isoelectric point, the solubility of γ -casein is approximately 2.5 times greater in water and 75 times greater in 50% alcohol than that of β -casein at room temperature. Formation of supersaturated solutions by γ -casein in water and in 50% alcohol is pronounced, and as a consequence, due consideration must be given to the time element in the study of solubilities and isolation procedures. The ability of γ -casein to form supersaturated solutions at an alcohol concentration approaching 95% or higher is shown by the continued precipitation, with time, of a 50% alcohol- γ -casein solution diluted with absolute alcohol. The solubility of γ -casein in 50% alcohol greatly increases at temperatures above 25° and decreases at lower temperatures. Conversely, the solubility of γ -casein in water is increased by lowering the temperature.

Isoelectric Point of γ -Casein.—By interpolation of the data in Fig. 2 to the point of zero mobility, the isoelectric point of γ -casein at an ionic strength of 0.05 can be estimated. The value of 5.8 agrees well with the value of about 5.8-6.0, determined by the point of minimum solubility and by the pH best suited for flocculation.

γ -Casein is electrophoretically homogeneous on the alkaline side of the isoelectric point, but it is heterogeneous on the acid side; it is analogous to α - and β -casein in this respect.⁶

Discussion

It is difficult to estimate the amount of γ -casein present in unfractionated casein from the electrophoretic pattern (Fig. 1 (a)). However, calculations based on the electrophoretic patterns of Fig. 1 (b) and (c) combined with the weights of these fractions indicate that unfractionated casein contains about 3% γ -casein. That γ -casein is a component of casein and not a constituent of whey protein precipitated with casein is indicated by the fact that no fraction of whey protein has been found with the electrophoretic mobility and properties of γ -casein. Since γ -casein is appreciably soluble in water, and is acid to the isoelectric point at pH 4.7, at which pH casein is removed, it might be expected that milk whey would contain significant quantities of γ -casein. In an attempt to prepare γ -casein from

whey, the proteins of whey were extracted with alcohol by the method used for preparing γ -casein. A small amount of whey protein was found to be soluble in 50% alcohol at room temperature. The electrophoretic mobility of the alcohol-soluble whey protein was greater, however, than that of γ -casein.

A further attempt was made to demonstrate the presence of γ -casein in whey by heat coagulating the proteins of whey at pH 4.7. It might be expected that γ -casein would be present in the filtrate after heat coagulation, since γ -casein is not coagulable by heat. Electrophoretic analysis of the protein of whey not coagulable by heat, however, did not reveal γ -casein. Osborne and Wakeman⁴ were unable to demonstrate the presence of their alcohol-soluble casein in whey by extraction of the whey proteins with alcohol, but they obtained anaphylactic reactions with the protein not coagulable by heat, which they interpreted as indicating the presence of their alcohol-soluble casein. The apparent complete removal of γ -casein in the precipitation of the casein fraction from skim milk at pH 4.7 may be in part explained by the effect of the salt present in decreasing its solubility, as was demonstrated by Linderstrøm-Lang and Kodama⁶ for unfractionated casein in solution acid to the isoelectric point and in part by its complex formation with α - and β -casein.

The sulfur and phosphorus contents of γ -casein (Table I) are approximately the same as those reported by Osborne and Wakeman for their alcohol-soluble casein. γ -Casein also resembles the alcohol-soluble casein in that its aqueous solutions become opaque when warmed. β -Casein solutions, however, behave in a similar manner. The sulfur contents of α - and β -casein are consistent with the estimates of the sulfur-containing amino acids reported by Gordon, *et al.*⁷

The optical rotations of the three casein fractions are consistent with the rotation (α)_D²⁵ -105 of unfractionated casein, based on the composition of unfractionated casein determined by electrophoretic analysis—approximately 75% α -casein, 22% β -casein and 3% γ -casein.

Acknowledgment.—We are indebted to C. L. Ogg of this Laboratory for the sulfur analyses.

Summary

A method is described for separating γ -casein from α - and β -casein that involves alcohol-water fractionation and isoelectric precipitations. In composition and properties, γ -casein is similar to the alcohol-soluble casein described by Osborne and Wakeman.

(7) Gordon, Semmett, Cable and Morris, *THIS JOURNAL*, 71, 3293 (1949).